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# Induced Endocytosis in Human Fibroblasts by Electrical Fields

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**Electroporation creates transient pores through which exogenous molecules can gain access to the cell cytoplasm. However, the electrical events associated with this phenomenon may perturb membrane-dependent events such as endocytosis. To measure the effect of electroporation on endocytosis, suspensions of human gingival fibroblasts were subjected to 5-ms electrical discharges, allowed to recover for variable periods of time, incubated with fluorescent probes, and then analyzed by flow cytometry. Incubation of electroporated fibroblasts with FITC-conjugated bovine serum albumin (BSA) to label moities on cell membranes nonspecifically demonstrated a time-dependent increase of internalized probe for up to 90 min after electroporation. Pretreatment incubation of cells with cytochalasin D abrogated the increased internalization of FITC-BSA due to electroporation. Compared to controls, fluorescence signals due to internalization of surface glycoproteins with FITC-concanavalin A were 43% higher after electroporation and treatment with endoglycosidase F or H to reduce probe associated with surface membrane. Confocal microscopy confirmed intracellular labeling and reduction of membrane-associated probe by the enzyme. Assessment of nonspecific FITC-Con A labeling of cells by pretreatment with α-methyl D-mannoside showed that labeling was largely (92%) specific. Compared to controls, electroporation induced a 60% increase of internalization of lucifer yellow, a fluid-phase endocytosis marker. Dual fluorescence labeling of membrane phosopholipids by FITC and TRITC-DHPE demonstrated an increased acidification after electroporation that was time dependent, indicating that electroporation induced more rapid entry of membrane lipid into endosomal compartments. These data demonstrate that the electrical fields used in electroporation of fibroblasts cause an actin-dependent increase in the internalization of all membrane components examined and an increased rate of probe entry in to acidifying compartments.** 

## INTRODUCTION

Electroporation uses electrical fields to create a transient breakdown in the plasma membrane. This loss of membrane integrity facilitates cell fusion and enables direct access to the cell cytoplasm for loading of various exogenous molecules. Plasmid DNA for transfection [1–3], complex carbohydrates [1], and proteins (including enzymes and antibodies) [4–7] have all been successfully introduced into cells using this method. However, it has been observed that electroporation may induce long-lasting perturbations of cellular metabolism and structure in addition to the creation of transient breakdowns in the plasma membrane. For example, altered cell membrane structure [8], cytoskeletal rearrangements, and membrane protrusions [9] have been observed following electroporation.

It has also been suggested that the electrical fields associated with electroporation induce more rapid uptake of exogenous probe by endocytosis [10]. This phenomenon may lead to inappropriate labeling of endosomal compartments instead of the intended cytoplasmic space [4, 10, 11]. Here we report the effect of electroporation on internalization of several components of the membrane (membrane lipid and glycoprotein) as well as fluid-phase endocytosis and nonspecific endocytosis of exogenous protein. The experiments demonstrate induced endocytosis by electrical fields.

## MATERIALS AND METHODS

*Cell preparation.* Human gingival fibroblasts (passage 5 to 15) originally derived from primary explants were grown as monolayer cultures in alpha minimum essential media containing 15% heat-inactivated fetal bovine serum (FBS, Flow Laboratories, MacLean VA), a 0.17% (w/v) penicillin solution, and a 0.1% (w/v) gentamycin sulfate solution (Sigma Chemical Co., St. Louis, MO). The cells were maintained in a humidified incubator ventilated with 95% air–5% $CO_2$, harvested by trypsinization, and counted electronically (Model ZM Coulter Counter, Coulter Electronics, Hialeah, FL). Cells were pelleted and resuspended at the desired concentration in electroporation buffer (1.26 m*M* $CaCl_2$, 5.37 m*M* KCl, 0.52 m*M* $KH_2PO_4$, 0.64 m*M* $MgCl_2$, 0.63 m*M* $MgSO_4$, 85.5 m*M* NaCl, 5.8 m*M* $NaHCO_3$, 0.50 m*M* $NaH_2PO_4$, 12.5 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Hepes).

*Probes and electroporation.* Fluorescein - 5 - isothiocyanate - conjugated bovine serum albumin (FITC-BSA), FITC-conjugated concanavalin A (FITC-Con A) and lucifer yellow were purchased from Molecular Probes (Eugene, OR); propidium iodide and cytochalasin D were purchased from Sigma. The probes were prepared at the indicated concentrations in electroporation buffer. An electroporation apparatus (Bio-Rad gene pulser, Bio-Rad, Mississauga, Ontario)

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with a capacitance expander and Gene Pulser cuvettes (0.4 cm interelectrode distance; Bio-Rad) were used for all experiments. An aliquot (800 μl) of $10^6$ cells was electroporated at 500 V/cm with a capacitance of 250 μF which produced a discharge time of 5 ms. Under these conditions, greater than 75% of cells are viable and are labeled with exogenous probe [4]. After electroporation, cells were allowed to recover for various time periods (5–120 min) at 37°C and were incubated with probe for 10 min. Cells incubated with FITC-BSA (1.89 μM) were then trypsinized for 5 min to remove surface bound protein. To label cell surface glycoproteins, cells were incubated with FITC-Con A (19 μM). In some experiments, cells incubated with Con A were treated with endoglycosidase H (20 μU per 60 μl of cells for 3 h at 37°C.; Boehringer Mannheim, Montreal, Quebec) or endoglycosidase F (0.5 μU per 60 μl of cells for 3 h at 37°C.; Boehringer Mannheim) after electroporation to reduce surface-bound label; these enzymes cleave *N*-glycans in cell surface glycoproteins that bind Con A [12, 13] In other experiments, cells were incubated with α-methyl pyrannoside ($8.5 \times 10^{-5}$ *M*; Sigma) before Con A to examine the specificity of Con A binding. Cell membranes were double labeled with FITC - 1, 2 - dihexadecanoyl - *sn* - glycero - 3 - phosphoethanolamine, triethylammonium salt (FITC-DHPE; Molecular Probes), and TRITC-DHPE prepared as unilamellar liposomes. The probes were dissolved in ethanol, sonicated, diluted with electroporation buffer, and sonicated again. The final stock concentration of probes was 4.6 μM. Cells were labeled by adding 15 ml of stock solution to a T-75 flask at 37°C for 2.5 h, washed twice with PBS, trypsinized, and washed again prior to analysis. To block actin assembly, cytochalasin D was used. A single cell suspension (800 μl) was incubated with 10 μl of a 1 m*M* stock solution of cytochalasin D (final concentration 12.5 μM) and maintained at 37°C for 10 min prior to electroporation.

*Analysis.* Cells were pelleted, resuspended in $Ca^{2+}$-, $Mg^{2+}$-free PBS, and analyzed by flow cytometry (FACStar Plus; Becton-Dickonson, Mississauga, Ontario) with laser excitation of 488 nm for FITC, 441 nm for lucifer yellow, and 514 nm for TRITC. Green fluorescence (FITC) was measured using a 530/30 nm filter, red fluorescence (TRITC) with a 585/42 nm filter, and the fluorescence due to lucifer yellow with a 530/30 filter. Two-color emission from FITC-DHPE- and TRITC-DHPE-labeled cells was collected simultaneously. All fluorescence signals were logarithmically amplified. Ten thousand cells were analyzed in each sample and data were collected in list mode. To eliminate signals due to cellular debris, only those events with forward scatter and side scatter comparable to whole cells were analyzed.

A standard curve for the pH-dependent emission of FITC-DHPE and the pH-independent emission of TRITC-DHPE was constructed using solutions of standard pH and a spectrofluorimeter (PTI, London, Ontario). For FITC-DHPE, excitation was set at 490 nm, emission was set at 530 nm, and slit widths were set at 4 nm. For TRITC-DHPE, excitation was set at 514 nm and emission at 580 nm and slit widths were set at 4 nm. Aqueous suspensions of TRITC and FITC-DHPE were analyzed over the pH range of 4–6.5.

Data described here are representative of at least two replicated experiments and the data are reported as the mean fluoresence channel number ± 95% confidence limits. Statistical comparisons between two experimental groups were performed by Student *t* test.

*Confocal microscopy.* To visualize the intracellular location of the probe, a single cell suspension of electroporated cells in phosphate-buffered saline (pH 7.4) was imaged using a 40× water immersion lens on a confocal microscope (excitation, 488 nm; emission, 530 nm; Bio-Rad; Ontario Laser and Lightwave Research Centre; University of Toronto; or Leica CLSM, Wetzlar, FRG). Computer-generated images of 0.5-μm optical sections were obtained at the approximate geometric center of the cell as determined by repeated optical sectioning.

## RESULTS

### *Membrane Integrity*

Whereas the level of fluorescence due to propidium iodide (PI; 1200 D; 10 μg/ml) staining of paraformaldehyde-fixed cells was 314.5 ± 1.96 (mean fluorescence channel numbers ± 95% confidence limits), the fluorescence after incubation with PI and no electroporation in viable, unfixed cells was 2.88 ± 0.14 while 1 min after electroporation in viable, unfixed cells the amount of PI internalized was ~24% lower than the positive control (PI present at time of electroporation in viable cells, 6.1 ± 0.15; 1 min after electroporation, 4.9 ± 0.13; 1 min PI with no electroporation, 3.6 ± 0.12). At 3 and 5 min, a similarly decreased level of PI incorporation was found (3 min, 5.3 ± 0.17; 5 min, 4.8 ± 0.16). This increased dye exclusion and membrane impermeability to small molecular mass compounds following electroporation was consistent with an earlier report [10] on trypan blue exclusion (molecular mass 961). Experiments employing a similar design were performed using FITC-BSA (molecular mass 66 kDa; 1.89 μM; 10 min incubation) to determine pore closure and the loss of membrane permeability to larger compounds that occurs following electroporation. After cold (4°C) incubation to block constitutive nonspecific endocytosis and a 5-min postelectroporation recovery period, electroporated cells demonstrated a similar level of probe uptake (2.72 ± 0.07) as the nonelectroporated samples (2.51 ± 0.03), which although marginally higher than autofluorescence (2.05 ± 0.02), was ~$2\frac{1}{2}$ times lower ($P < 0.01$) than cells electroporated in the presence of FITC-BSA (6.56 ± 0.19). Thus the data indicate that for the longer recovery periods (5 min and greater) used in the experiments, ~95% of the fluorescence signal was attributable to loading via mechanisms other than membrane pores created by electroporation.

### *Nonspecific Endocytosis*

After recovery periods of 5 to 120 min, cells were incubated with FITC-BSA for 10 min to evaluate the effect of electroporation on nonspecific endocytosis. To eliminate surface-bound probe that was not endocytosed prior to analysis, cells were trypsinized (0.01% bovine trypsin for 5 min at 37°C). After 10-min incubations with FITC-BSA, trypsinization decreased cellular fluorescence by nearly sixfold (Fig. 1). Shorter incubation times with FITC-BSA of 1 min were then used to reduce constitutive endocytosis and these data demonstrated a twofold reduction of fluorescence for incubations at 37°C (28.1 ± 0.74, no trypsinization; 14.8 ± 0.75, trypsinization) while for incubations at 4°C there was only a small additional reduction of fluorescence (12.7 ±

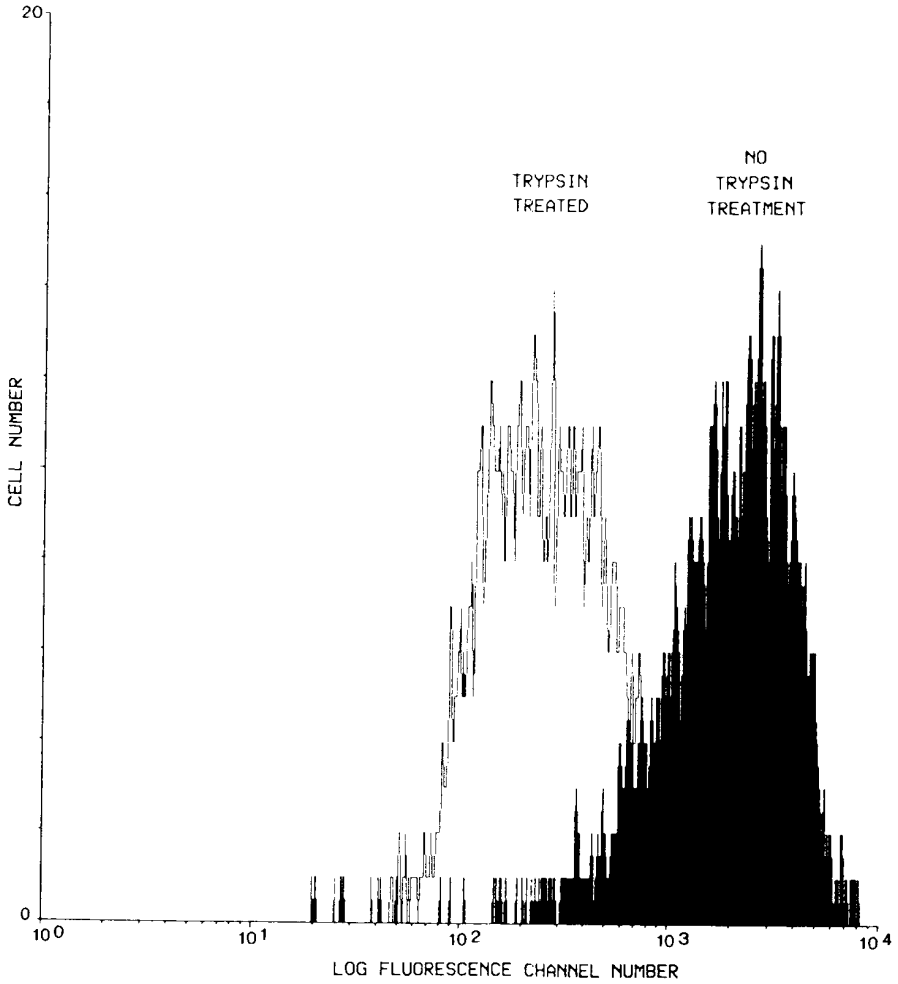


**FIG. 1.** Single parameter flow cytometric histograms demonstrating the effect of postelectroporation trypsinization on reduction of signal attributable to membrane-bound FITC-BSA. Electroporated fibroblasts (500 V/cm, 250 $\mu$F) incubated with FITC-BSA (1.89 $\mu M$) for 10 min followed by trypsinization (0.01%) at 37°C.

1.0). Optical sections (nominal 0.5 $\mu$m) obtained by confocal microscopy demonstrated that before trypsinization, FITC-BSA was largely confined to the cell membrane (Fig. 2a), whereas after trypsinization, most of the probe was restricted to the cell interior and very little probe was detectable on surface membranes (Fig. 2b). Subsequent experiments employing trypsinization to reduce membrane-associated probe demonstrated that in comparison with controls (not electroporated and incubated with probe for 10 min; mean fluorescence channel number, 214 ± 3.7), electroporated cells exhibited double the fluorescence 10 min after electroporation (487 ± 18.3). Thirty minutes after electroporation there was 2.5- to 3-fold more fluorescence in electroporated samples (682 ± 19.6) than that in nonelectroporated samples and this increased to 4-fold higher levels at 90 min (827 ± 25.6). Because the cells survive for only ~90 min in suspension (as demonstrated by plating efficiency and reduction of forward light scatter-FSC; control cells, 705 ± 6; 5-min recovery, 705 ± 6; 90 min recovery, 593 ± 4; Fig. 3), it was not possible to determine when or if endocytosis returned to basal levels.

*Fluid-Phase Endocytosis*

Cells that were electroporated, allowed to recover for 5 min, and incubated with lucifer yellow (molecular mass 457; final concentration 2.73 m$M$) for 5 min exhibited 60% higher fluorescence ($P < 0.001$) than that of control cells incubated only with the probe (mean fluo-

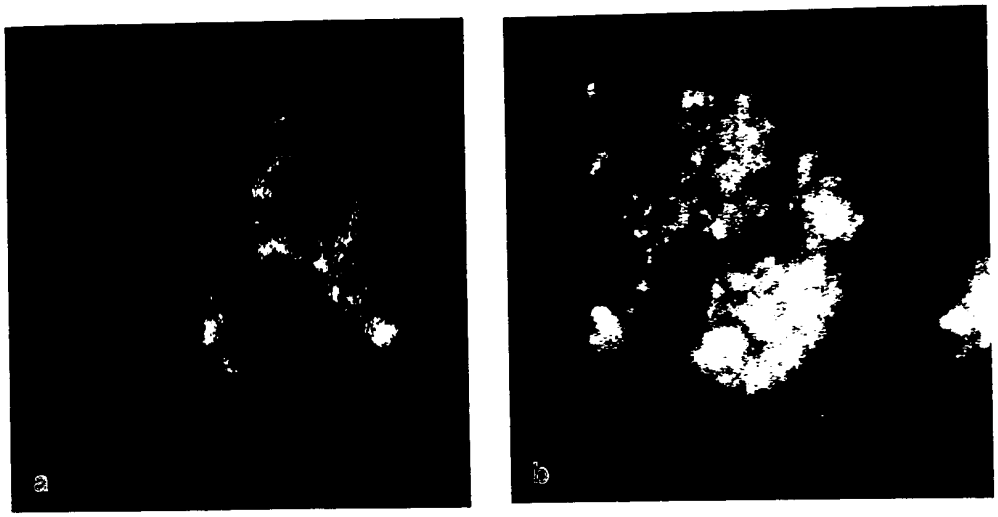


**FIG. 2.** (a) Optical section (nominal thickness 0.5 $\mu$m) obtained by confocal microscopy of an electroporated fibroblast incubated with FITC-BSA and without trypsinization demonstrating FITC-BSA label on the cell membrane. (b) Optical section of an electroporated fibroblast after FITC-BSA incubation and trypsinization showing label in the cell interior and in localized vesicular bodies but only low levels of membrane-associated probe after trypsinization. Electroporation appears to induce polar distribution of probe, perhaps indicating the orientation of the cell during the transient electrical pulse (×3000).

rescence channel numbers: 12.3 ± 0.44, electroporation (el); 7.09 ± 0.48, control, no electroporation (no-el); 2.03 ± 0.07, autofluorescence). This increased rate of fluid phase endocytosis was consistently higher in the electroporated samples at 15 min (10.1 ± 0.75, el; 6.81 ± 0.21, no-el; $P < 0.001$) and 60 min after electroporation (13.1 ± 2.11, el; 7.27 ± 1.36, no-el; $P < 0.001$)).

*Membrane Glycoproteins*

Blocking surface receptor binding sites for Con A with $\alpha$-methyl pyrannoside ($8.5 \times 10^{-5}$ $M$; 10 min) prior to electroporation and incubation with FITC Con A (19 $\mu M$; 10 min) sharply reduced (by 92%) the signal attributable to surface-bound and endocytosed probe, indicating that FITC-Con A binding to cell surface glycoproteins was largely specific (no incubation, 1161 ± 40; preincubation, 93 ± 5). However, FITC-Con A could not be completely removed from cell membranes by trypsinization (Fig. 4a), presumably due to the resistance and inaccessibility of integral membrane proteins for enzyme cleavage. Because the flow cytometer measures whole cell fluorescence and cannot distinguish surface-bound from internalized probe, several methods of reducing surface-bound probe were used to assess more

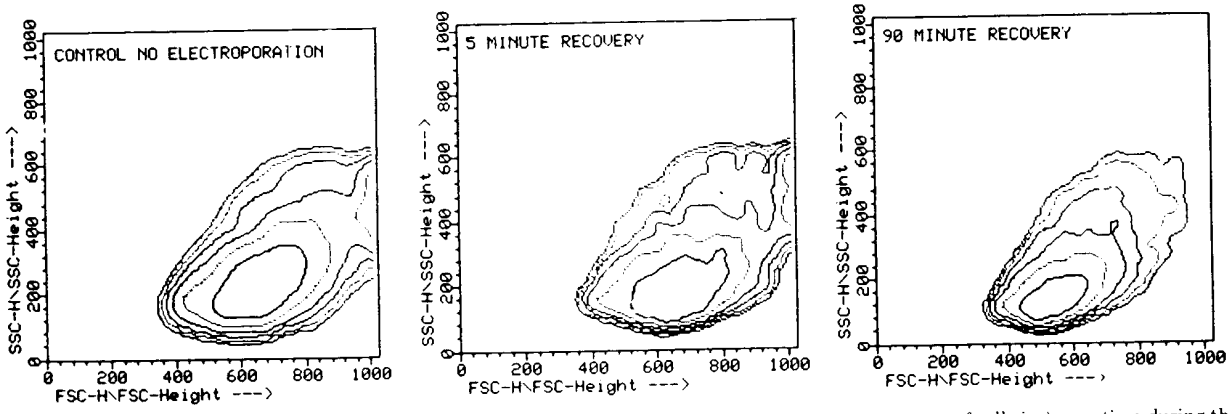


**FIG. 3.** Bivariate flow cytometric contour plots demonstrating loss of forward light scatter (a measure of cell size) over time during the recovery period; note that side scatter (a measure of cytoplasmic granularity) remains relatively constant.

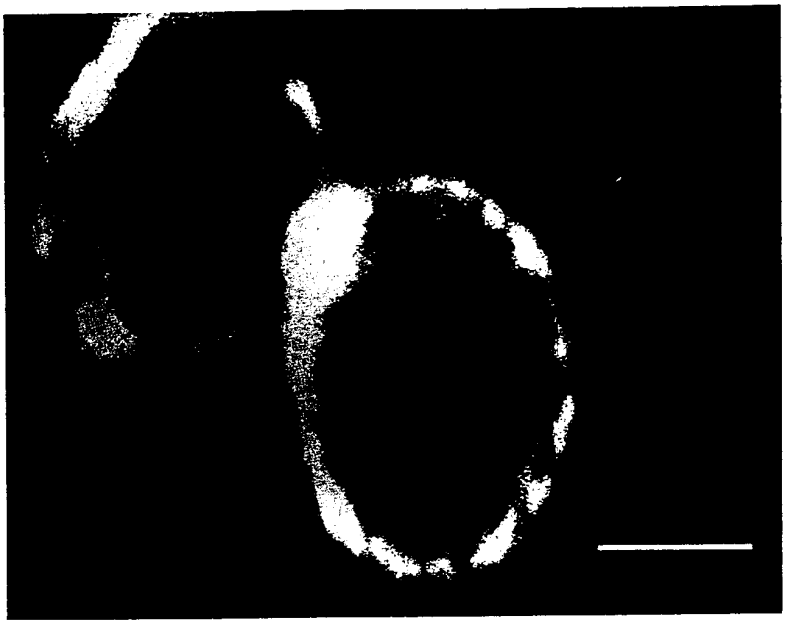

**FIG. 4a.** Confocal micrograph of a fibroblast incubated with FITC-Con A and then trypsinized demonstrating bright fluorescence due to remaining, membrane-associated probe (×2200; scale bar, 10 μm).

accurately the contribution of internalized probe to the overall fluorescence signal.

The enzymes endoglycosidase H and F hydrolyze *N*-glycans of the high mannose type [12, 13]. Enzyme treatments of 3 h at 37°C were used to reduce surface-bound FITC-Con A prior to analysis. Enzyme-treated samples exhibited a significantly ($P < 0.001$) reduced fluorescence compared with untreated samples (enzyme treated, 900 ± 130; no enzyme, 2806 ± 87; ~66% decrease in fluorescence). Following electroporation, FITC-Con A incubation, and endoglycosidase F treatment, cells exhibited a 43% increased signal attributable to Con A endocytosis compared to nonelectroporated samples: the electroporated population treated with endoglycosidase F exhibited a mean fluorescence of 1411 ± 79 while nonelectroporated samples exhibited a mean fluorescence of 990 ± 130. Electroporated samples treated with endoglycosidase H exhibited a 47% increase after electroporation (el, 1963 ± 85; no-el, 1337 ± 78).

A second approach to estimate the amounts of internalized and membrane-bound probe was by the use of confocal microscopy. Optical sections through the center of enzyme-treated cells demonstrated bright cytoplasmic fluorescence in electroporated samples (Fig. 4b), and scanning fluorimetry of digitized images through the cell center indicated that in some cells up to ~20-fold higher fluorescence signals were measured inside the cell than those of the cell membrane. Collectively, these two methods of assessing internalized FITC-Con A-labeled moities indicate that electroporation induces increased internalization of cell surface glycoproteins.

*Membrane Internalization*

Cells labeled with FITC-DHPE and TRITC-DHPE and then electroporated were analyzed by two-color flow cytometry to assess the rate of acidification of probe in presumptive endocytic compartments. Computation of the ratio of FITC fluorescence (pH dependent) to TRITC fluorescence (pH independent) permitted an estimation of the pH environment of the overall probe signal, independent of cell size and degree of probe loading. Analysis of a standard curve indicated that over the pH range of 4.0–6.5, the dual labeling method was sensitive to pH change (Fig. 5a). Electroporated cells showed a statistically significant lower ratio of FITC to TRITC fluorescence at 10, 30, and 60 min ($P < 0.05$) than that of nonelectroporated samples (Fig. 5b) while at 90 min, the ratios were the same for both experimental and control groups.

*Dependence on Actin Assembly*

Cells preincubated with cytochalasin D (15.5 μ*M*; 10-min treatment) prior to electroporation and followed by

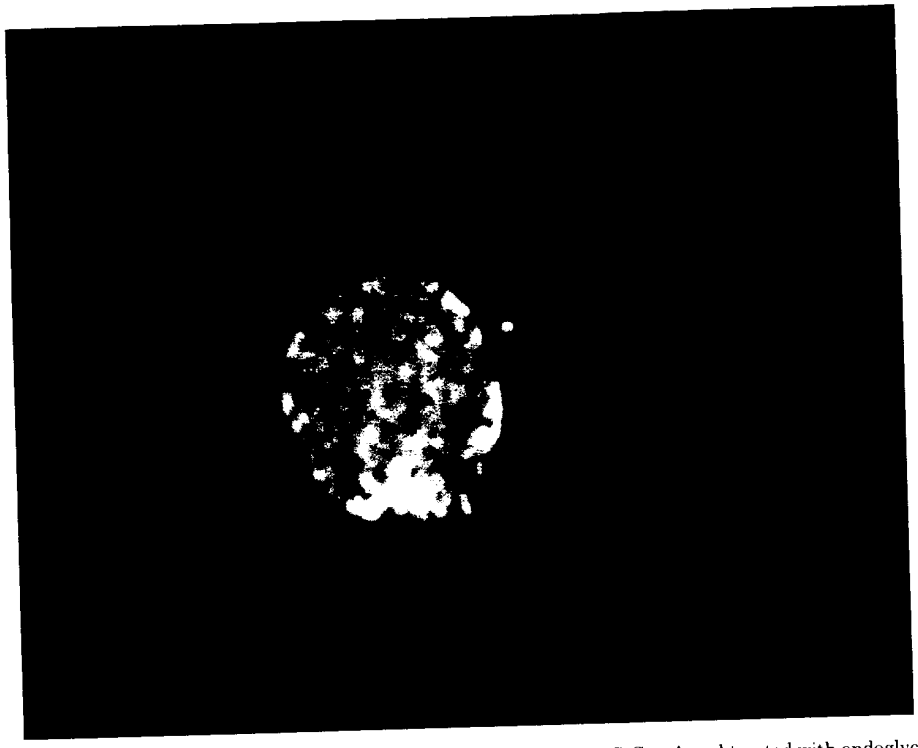

**FIG. 4b.** Confocal micrograph of an electroporated fibroblast incubated with FITC-Con A and treated with endoglycosidase F. Note the bright fluorescence labeling within the cell and the relative absence of membrane-associated probe (×1320).

a 10-min recovery period demonstrated a 79% reduction ($P < 0.01$) of FITC-BSA labeling (8.86 ± 2.46) compared to electroporated cells which were not incubated with cytochalasin D (41.74 ± 1.89).

## DISCUSSION

### *Labeling of Subcompartments*

We have described the effect of electrical fields on endocytosis in fibroblasts. The data demonstrate that cells exposed to a short duration (5 ms) electrical field exhibit an increased rate of endocytosis. However, the method of measuring endocytosis of fluorescent probes involved analysis of whole cell fluorescence and introduced the possibility of artifactual estimates. For example after electroporation, labeled probe could be associated with any one or more of the cytosolic, surface membrane and endosomal compartments. Consequently a series of experiments was conducted to estimate the contribution of label in those compartments to the whole cell fluorescence signal and to evaluate methods for minimizing signal in the cytosol and that associated with surface membrane components.

Introduction of label into the cytosol is believed to occur by transient pore formation, a result of mechanical compressions of the membrane created by the electrical field [13]. To assess the duration of loading via pores, the time prior to resealing must be known. Zimmermann *et al.* [10] found that 90% of electroporated mouse L-cells exclude trypan blue within a few seconds and electron microscopic studies have indicated loss of detectable pores 10 s after electroporation [8]. We found that after a 5-min recovery following electroporation and a subsequent FITC-BSA (molecular mass 66 kDa) incubation, the amount of probe internalized through pores was reduced by ~95% when compared to cells electroporated in the presence of FITC-BSA. These data indicate that the amount of cytosolic loading through pores is negligible by 5 min when compared to the level of endocytosed probe.

Probe associated with the surface membrane was removed with postincubation, preanalysis enzyme treatments. The effectiveness of the enzyme treatments was demonstrated by the large-scale reduction of membrane-bound probe when examined by confocal microscopy and flow cytometry. Notably, BSA was largely removed by trypsinization while endoglycosidase F and H were both effective in reducing Con A binding to surface membrane that was not endocytosed. Consequently,

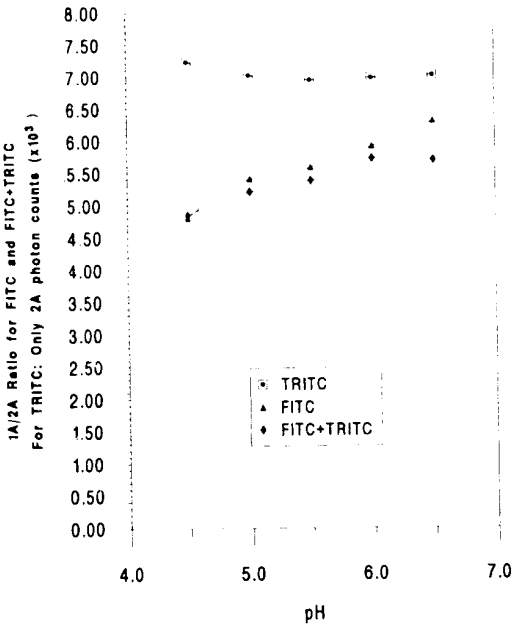


**FIG. 5a.** Standard curve of pH-independent fluorescence emission of TRITC-DHPE and the pH-dependent emission of FITC-DHPE measured by spectrofluorimetry.

membrane labeling did not contribute substantially to the whole cell fluorescence signal. We [4] and others [5, 10] have demonstrated entry of fluorescence probes into vesicular, low pH, presumptive endosomal compartments. Based on the vesicular distribution of labeled probe observed in confocal micrographs and the pH decrease following labeling, we conclude that after a short recovery period to allow for pore closure and appropriate enzyme treatments, flow cytometry provides an accurate estimate of the relative amounts of exogenous label in endocytic compartments in electroporated and nonelectroporated cells.

*Internalized Components of the Membrane*

The first set of experiments used FITC-BSA as a probe to determine if electroporation increased the rate of nonspecific endocytosis. Previous data have indicated that this probe may be internalized into vesicular compartments [4–6, 10] and our confocal micrographs confirmed the presence of probe in vesicles as early as 5 min after electroporation. Although flow cytometric estimates of internalized probe may have been reduced because of intracellular and pH-dependent fluorescence quenching which occurs within 5 min of endosome formation as the vesicle interior acidifies [14], our analysis of electroporated cells by flow cytometry demonstrated a statistically significant and time-dependent increase in the amount of internalized probe that was two- to fourfold higher than that of controls.

Fluid-phase endocytosis involves the continuous uptake of extracellular fluid through small membrane vesicles. Fluid-phase vesicles by definition are labeled by impermeable solutes which do not bind to the cell's membrane [15]. Uptake of lucifer yellow, a fluid-phase marker, was also increased up to 60% in electroporated cells compared to controls. We conclude therefore that electroporation upregulates both fluid-phase and nonspecific endocytosis in suspended fibroblasts but that the measureable effect is relatively larger for nonspecific endocytosis. This is not surprising because FITC-BSA is not solely a marker of fluid-phase endocytosis. However, this result suggests also that electroporation may create substantial perturbations in membrane domains. Indeed, the apparent membrane intercalation of FITC-BSA observed in some confocal micrographs even after trypsinization indicates that FITC-BSA cannot be wholly removed from deeper membrane components by trypsinization, indicating that FITC-BSA-labeled membrane domains are subsequently internalized and may contribute to the overall increased cellular fluorescence with this probe. In contrast, the relatively

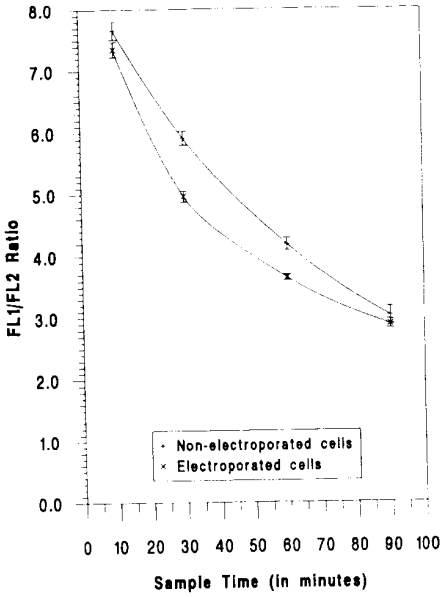


**FIG. 5b.** Ratio of FITC to TRITC-DHPE signal (proportional to pH) with associated 95% confidence limits. Over time, electroporated and nonelectroporated fibroblasts exhibit different kinetics of probe acidification. Electroporated cells exhibit more rapid ($P < 0.05$) acidification of internalized phospholipid probe than that of controls at 10, 30, and 60 min after electroporation.

lower induction of fluid-phase endocytosis by electroporation as detected by lucifer yellow could reflect increased intracellular quenching of this dye compared to that of FITC-BSA.

To study internalization of lipids associated with surface membranes, FITC and TRITC-DHPE were incorporated into cells prior to electroporation. Analysis by flow cytometry demonstrated that labeled phospholipids entered acidic compartments more rapidly in electroporated cells than controls. Uptake of membrane lipid into induced endosomes is consistent with the data of Chenomordik *et al.* [16] who showed that DNA introduced into cells by electroporation was taken up by vesicles with lipid membranes. Our data demonstrate in addition that the endocytosed lipid is derived at least in part from the cytoplasmic membrane. Because the internalized membrane is a representative sample of the plasma membrane [15], internalized labeled phospholipid may originate from sites of membrane damaged by the electrical field [17].

To determine if membrane-associated glycoproteins were involved in electrically induced endocytic events, FITC-Con A was used to label surface glycoproteins after recovery from electroporation. Due to the difficulty of removing surface-bound Con A by trypsinization, two experiments using alternative approaches were conducted to estimate the amount of internalized probe. Data from enzyme-treated cells indicated that labeling due to internalized probe was higher in electroporated cells than that in controls and confocal microscopy demonstrated that vesicular structures strongly labeled with Con A were more rapidly formed. Taken together, we conclude that electroporation induced a globally increased internalization of membrane lipid, membrane glycoproteins, and other structures labeled with BSA into lipid-delimited, acidifying vesicles.

### *Mechanisms of Induced Endocytosis*

Several studies have demonstrated the development of membrane ruffling after the exposure of cells to electrical fields [6, 8] and increased ruffling and vesicle formation may persist for up to 4 h after field exposure [6]. Because pinocytosis and endocytosis induced by growth factors [18] and phorbol esters [19] are associated with the initiation of membrane ruffling [20], it is conceivable that electrical field-induced membrane perturbations such as breakdown of membrane continuity and creation of membrane fatigue [17] could account in part for the increased ruffling and the increase in endocytosis. These responses may involve an attempted repair of denatured membrane protein. Tsong [17] has suggested that electroporation produces localized joule heating of the membrane which denatures membrane proteins such as voltage- and ligand-dependent protein and ion channels. Following denaturation, it has been proposed that damaged patches of membrane are excised by endocytosis [17]. This mechanism is consistent with the observed increase of endocytosis after electroporation and the more rapid acidification of fluorescent phospholipids presumably derived from the membrane domain.

Large-scale shifts of intracellular ion and protein concentrations caused in part by sodium and potassium ion leakage through lattice defects in the lipid bilayer [21] may induce severe osmotic stresses which affect cytoskeletal structures [8] and cell size, consistent with our observation of reduced forward light scatter in electroporated cells. An alternative mechanism may involve a clathrin-independent endocytic pathway [20] in which the constitutive clathrin pathway is blocked by electroporation. This poorly understood pathway may be invoked by electroporated cells as a stress response to compensate for the loss of intracellular molecules induced by the electrical field. Whatever mechanism is invoked, it is evident that electroporation perturbs both membrane- and cytoskeleton-dependent elements of endocytosis. Indeed we observed that cytochalasin D inhibited the electroporation-induced increase of endocytosis, presumably by blockade of actin assembly from monomer. Previous studies have demonstrated a direct perturbation of the cytoskeleton by electrical fields [9] and electroporated erythrocytes exhibit pores of 40–100 nm in diameter that are the same dimension as the spectrin, ankyrin, and actin skeletal network [22]. Thus pores may reach a maximim size that is determined by the cytoskeleton and exhibit shapes and closure dynamics that are cytoskeleton dependent [8]. Consequently the observed inhibition of endocytosis by cytochalasin D is consistent with the view that electroporation-induced endocytosis is dependent on actin rearrangement.

Our results indicate that in addition to the utility of electroporation for introduction of exogenous molecules, study of electrical field effects and the accompanying physical forces that are delivered to the cell can be used to probe endocytosis and the dynamic interactions of plasma membrane proteins with the cytoskeleton [23].

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